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In Vitro Neutralization of Hepatitis B Virus by Monoclonal Antibodies Against the Viral Surface Antigen

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In vitro HBV infection and neutralization were assayed using an anti-preS1 murine monoclonal antibody (1B3) and anti-preS2 (H69K) and anti-S (CS131A) murine-human chimeric antibodies. The 1B3 (IgG1) and H69K (IgG1) was constructed previously and the CS131A was constructed for this study by expressing stably the chimeric heavy and light chains in Chinese hamster ovary cells and purifying from the culture supernatant. Previous study showed that the H69K and CS131A recognize known virus-neutralizing epitopes, while the 1B3 does not. For the assays, adult human hepatocyte primary culture was infected with the *adr* or *ayw* subtype of HBV, and the infectivity and subsequent replication was confirmed both by measuring the kinetics of HBsAg secretion by the infected cells and detecting the intermediate replicative form of HBV DNA in the cells. Next, the hepatocytes were infected with the *adr* or *ayw* subtype of the virus that had been preincubated with various concentrations of each of the antibodies and the neutralization of HBV was analyzed. The results showed that the anti-preS2 and anti-S chimeric antibodies exhibited neutralizing activity against both the *adr* and *ayw* subtypes of the virus, with approximately 1,000 and 2,000 times higher specific activity than polyclonal hepatitis B immune globulin, respectively, but the anti-preS1 antibody scarcely neutralized the infection. The neutralizing activities of the antibodies were consistent with their epitope specificity and antigen-binding affinity, suggesting that this neutralization assay is specific. The in vitro neutralization assay will be useful for evaluating the neutralizing activity of anti-HBV antibodies before in vivo testing in chimpanzees. ***J. Med. Virol. 52:226–233, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: in vitro HBV infection; neutralization; monoclonal antibodies; anti-preS1; anti-preS2; anti-S prophylaxis

INTRODUCTION

Hepatitis B virus (HBV) is a worldwide public health problem affecting many millions of people. For the prevention or post-exposure prophylaxis of HBV infection, hepatitis B immune globulin (HBIG) prepared from pooled human anti-HBsAg plasma is administered at birth to infants born of HBsAg-HBeAg positive mothers, susceptible individuals with acute exposure to infectious HBV-containing material, or orthotopic liver transplant patients with chronic HBV-related liver disease [Beasley et al., 1983; Todo et al., 1991]. However, the currently available HBIG is not an ideal source of antibody due to its limited availability and low specific activity. Therefore, production of human monoclonal antibodies (mAbs) or murine-human chimeric antibodies to reduce the immunogenicity of the murine antibody in humans, with specificity for the surface antigens of HBV, have been reported for immunoprophylaxis or the treatment of HBV infection and disease [Harada et al., 1989; Li et al., 1990; Jin et al., 1993; Ogata et al., 1993].

The neutralizing and protective efficacies of the antibodies directed against the surface antigen have been evaluated in chimpanzees, but this is very expensive and time consuming [Iwarson et al., 1985; Itoh et al., 1986; Neurath et al., 1986; Harada et al., 1989; Ogata et al., 1993]. This is due to the difficulties not only in in

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vitro infection of cell lines by HBV but also in obtaining human adult hepatocytes and maintaining liver-specific functions for a prolonged period of culture. These technical difficulties have so far hampered the prediction of the neutralizing activity of anti-HBV monoclonal or polyclonal antibody as well as the elucidation of the nature of HBV infection. Recently, reproducible in vitro infection of adult human hepatocyte primary culture by HBV was demonstrated [Gripon et al., 1993], and this system may be useful for the evaluation of the neutralizing activity of the anti-HBV antibodies before the in vivo test.

The HBV envelope consists of 3 related proteins called the major, middle, and large proteins, and the proteins contain 3 different surface antigens, preS1, preS2, and S [Heerman et al., 1984]. All these antigens were shown to elicit antibodies that protect chimpanzees from HBV infection [Gerin et al., 1983; Purcell et al., 1985; Itoh, et al., 1986; Neurath et al., 1986, 1989]. Previously, we had generated murine mAbs with specificity for preS1, preS2, and S antigens, determined their epitope specificity, and subsequently constructed the anti-preS2 chimeric antibody for the prevention of HBV infection [Hong et al., 1992; Jin et al., 1993; Ryu et al., 1994; Kim et al., 1996]. In this study, we constructed anti-S chimeric antibody and explored in vitro neutralization of HBV infectivity by the anti-preS1, preS2, or S antibodies using in vitro infection system.

MATERIALS AND METHODS

Cell Culture

Murine hybridoma and transfectoma were cultured routinely at 5% CO_2, 37°C in IMDM (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco). The dihydrofolate reductase (dhfr)-deficient Chinese hamster ovary (CHO) cell line DG44 was kindly provided by Dr. Lawrence A. Chasin (Columbia University) and used for the expression of the chimeric antibody. The DG44 cells were grown in 5% CO_2 at 37°C in DMEM/F12 medium (Gibco) supplemented with hypoxanthine (10 μg/ml), thymidine (10 μg/ml), glycine (50 μg/ml), glutamine (587 μg/ml), glucose (4.5 mg/ml), 10% FBS, and antibiotic-antimycotic (Gibco). For the selection of the $dhfr^+$ transformants, the DG44 cells were grown in nucleosides-minus MEM α medium (Gibco) supplemented with G418 (550 μg/ml) and 10% dialyzed heat-inactivated FBS (Gibco). HepG2 (human hepatoma cell line) cells were cultured in H medium supplemented with 5×10^{-7} M hydrocortisone hemisuccinate and 10% porcine serum. H medium contains 75% minimal essential medium (Gibco) and 25% medium 199 (Gibco), insulin (5 μg/ml), bovine serum albumin (BSA, 1 g/l), penicillin (4.5 mg/ml), and streptomycin (50 mg/ml). Adult human hepatocytes were prepared by enzymatic dissociation of noncancerous liver fragments [Guguen-Guillouzo and Guillouzo, 1986]. All experimental procedures were in compliance with French laws and regulations and were approved by the National Ethics Committee. Freshly isolated hepatocytes were cultured in Williams' E medium (Gibco) supplemented with 5×10^{-6} M hydrocortisone hemisuccinate, 2% dimethyl sulfoxide, 5 μg/ml insulin, and 10% porcine serum.

Antibody Purification

Hybridoma and transfectomas were grown in serum-free medium (PFHM II and CHO-S-SFM, II, Gibco) and the culture supernatant was subjected to affinity chromatography on Protein G-Sepharose 4B column (Pharmacia) pre-equilibrated with 0.1 M sodium phosphate buffer (pH 8.0). The bound antibody was eluted with 0.1 M glycine HCl buffer (pH 2.7). The eluate was immediately neutralized with 1.0 M Tris (pH 9.0) and dialyzed against PBS (pH 7.0). For the quantitation of the purified antibody solution, the OD of 1.43 at 280 nm was taken for the protein concentration of 1 mg/ml [Coligan et al., 1991].

ELISA Analysis

The culture supernatant of the drug-resistant cells was analyzed in 96-well microtiter plates by an indirect ELISA [Engvall and Perlman, 1972]. For the detection of murine mAb and chimeric antibody, samples were added to each well which had been previously coated with the appropriate antigen and incubated at 4°C overnight. After washing, 100 μl of goat anti-human (or mouse) IgG peroxidase conjugate (1:1000 v/v, Sigma) was added to each well and incubated for 1 hr at 37°C. Finally, 100 μl of 0.2 M citrate-PO_4 buffer (pH 5.0) containing 0.04% o-phenylenediamine (Gibco) and 0.03% H_2O_2 was added to each well and incubated for 10 min. The reaction was stopped with 2.5 M H_2SO_4, and the OD was measured at 492 nm in an ELISA reader (Titertek Multiskan Plus).

For the determination of affinity, a solution containing 3 ng of antibody and various concentrations of antigen (10^{-12}–10^{-6} M) was first incubated at 37°C for 2 hr and then added to each well coated previously with 1 μg of the antigen. The concentrations of free antibody were determined by the indirect ELISA. Apparent affinity was determined as the reciprocal of the antigen concentration required to inhibit 50% maximal binding by competitive ELISA.

Construction of Chimeric Heavy and Light Chain Genes and the Expression Plasmids

Using recombinant polymerase chain reaction (PCR), the DNA sequence encoding the heavy chain variable region (V_H) or light chain variable region (V_L) of H67 [Ryu et al., 1994] was fused to the murine heavy chain or light chain leader sequence, which was then jointed to human Cγl or Cκ sequence in pCHC-S2 or pCKC-S2 [Jin et al., 1995], respectively. The resulting chimeric heavy chain cDNA was subcloned into EcoRI-SalI sites of pBluescript SK(+) to yield pCHC-S, and the chimeric light chain cDNA was subcloned into the HindIII-SalI sites of pBluescript SK(+) to yield pCKC-S.

To construct the chimeric heavy chain expression plasmid, pCHC-S was digested with SalI and the cohe-

sive ends were filled by Klenow enzyme. After further digestion with NotI, the resulting fragment was subcloned into pRc/CMV (In vitro gen) to yield expression plasmids pRc/CMV-HC-S.

To construct the chimeric light chain expression plasmid, the enhancer elements within the SV40 early promoter of pSV2dhfr [Subramami et al., 1981] were initially removed by deleting the sequence between SphI and PvuII sites at positions 128 and 270, respectively, followed by joining these sites via a synthetic BamHI linker. The crippled dhfr expression unit on a 1.6 kb BamHI fragment was subcloned into the BglII site of the pRc/CMV. The plasmid in which orientation of the transcription of the drug-resistant gene was opposite to that of the HCMV (human cytomegalovirus) promoter was selected and named pCMV-dhfr. The cDNA insert from the pCKC-S encoding the chimeric kappa chain was subcloned into pCMV-dhfr to yield expression plasmid pKC-dhfr-S.

Transfection and Selection

Five μg of each of the heavy and light chain expression plasmids were cotransfected into DG44 cells using 30 μg of Lipofectin (Gibco) according to the conditions recommended by the manufacturer. After selection in MEM α medium containing G418 in 96-well plates, the culture supernatant of the resistant cell clones was screened for the assembled antibody production by an indirect ELISA. Several high producer clones were isolated and grown individually in the selection medium containing 20 nM methotrexate (MTX, Sigma) in 10-cm culture dish to select for the amplified cell lines. After 2 to 3 weeks, the colonies that appeared on the dish were pooled and the productivity of the cells was determined by sandwich ELISA using polyclonal anti-human IgG. The amplified cell lines were subjected to the next round of MTX selection. This process was repeated with 4-fold increase in the MTX concentration until the final level of the MTX concentration reached 1 μM.

Preparation and Immunoprecipitation of HBV Particles

A replication competent and terminally redundant HBV-plasmid pHBV5.2 was constructed from pHBV-315 [*adr* subtype, Kim and Kang, 1984;]. This plasmid contains 3.2 kb HBV genomic DNA plus 2.0 kb BamHI-XhoI fragment of the genomic DNA in pBluescript SK(+).

To produce the virus particles, 3×10^7 HepG2 cells were transfected with 30 μg of pHBV5.2 by electroporation using Gene Pulser (Biorad) and cultured in H medium for 15 days. HBV particles were collected from the culture supernatant of the transfected cells by precipitation at 4°C for 12 hr with PEG 6,000 to a final concentration of 6%, followed by centrifugation at 10,000 × g for 30 min. The virus particles were suspended in 1/50 volume of PBS containing 25% fetal calf serum and stored at −70°C. The *ayw* subtype of HBV particles was collected from the culture supernatant of 2.2.15 [Sells et al., 198] and concentrated 100-fold.

The *adr* or *ayw* subtype of virus particles were immunoprecipitated overnight at room temperature with purified anti-preS1, preS2, or S antibodies (10 μg) bound to Protein A-Sepharose (Pharmacia). After washing the virus pellets 5 times with PBS, tRNA (10 μg) was added to the immunocomplex and the viral DNA was extracted [Hirt, 1967]. The viral DNA was analyzed by Southern blot hybridization using [α^{32}P]-labeled HBV genomic DNA as a probe [Southern, 1975]. Hybridization and washing were carried out at 65°C.

HBV Infection and Neutralization

To infect the human hepatocytes by the *adr* subtype of the virus, freshly isolated hepatocytes were seeded at a density of 10^6 cells per well containing 2 ml of normal growth medium. Three days after seeding, the cells were infected with about 3×10^7 viral genomic equivalent of the virus particles, as previously described [Gripon et al., 1993]. Hepatocytes were covered with 1 ml of culture medium (without serum) containing 4% PEG and inoculum. To infect the cells by the *ayw* subtype of the virus, 2×10^6 cells were infected by the same number of HBV particles. The infected cells were washed 3 times with the growth medium and further cultured for 15 days with the medium being changed every day. Secretion of HBsAg was assayed by a radioimmunoassay kit (Abbott Laboratories). A signal to noise (P/N) ratio of >2.1 was considered positive.

For a neutralization assay, various concentrations of the antibody, human IgG (Sigma), or HBIG (Korea Green Cross Co.) were preincubated with the virus particles at room temperature for 1 hr and inoculated onto the cultured hepatocyte. After 15 days, the HBsAg concentration of the culture supernatant was determined, and the intracellular viral DNA was extracted from the infected human hepatocytes [Hirt, 1967] and subjected to the Southern blot hybridization.

RESULTS

Murine or Chimeric mAbs Against the PreS1, PreS2, or S Antigen

Previously, we had generated and characterized 3 murine mAbs against the preS1, preS2, or S surface antigen. Anti-preS2 antibody H8 (γ2b, κ) recognizes the amino acids 13–24 of preS2 antigen [Hong et al., 1992] and is presumed to neutralize HBV because this epitope is known to elicit protective antibodies [Itoh et al., 1986]. Anti-S antibody H67 (γ1, κ) recognizes a disulfide-bond–dependent conformational epitope on the *adr* and *ayw* subtypes of HBsAg [Ryu et al., 1994], indicating that H67 recognizes a common *a* antigenic determinant. Since the common *a* determinant is known to induce virus-neutralizing and protective antibodies, H67 is also considered a neutralizing antibody. Anti-preS1 antibody 1B3 (γ1, κ) binds to the N-terminal 20 amino acids (M-G-G-W-S-S-K-P-R-Q-G-M-G-T-N-L-S-V-P-N) of longer version of HBV preS1

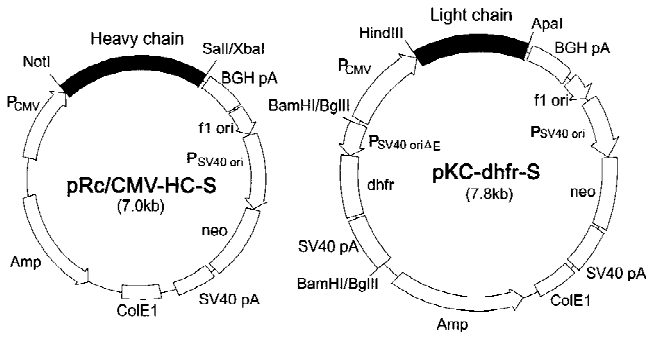

Fig. 1. The chimeric heavy (pRc/CMV-HC-S) and light (pKC-dhfr-S) chain expression plasmids. The cDNAs encoding the heavy and light chains are represented by the shaded boxes. Arrows indicate the orientation of the transcription.

antigen that was derived from *adr* subtype and expressed in *Escherichia coli* in a glutathione S-transferase fusion protein [Kim et al., 1996]. However, the preS1 region (amino acids 12–47) was known to elicit the neutralizing and protective antibodies by blocking virus attachment to cells [Neurath et al., 1989]. Therefore, the antibodies against the preS2 and S antigens were changed into the chimeric antibodies with murine variable and human constant regions to apply to the prophylaxis of HBV infection, since the chimeric antibodies not only reduce the immunogenicity in humans but also interact more effectively with the human immune system by virtue of selected human constant region [Morrison, 1985; Sun et al., 1987; Beidler et al., 1988; Hoogenboom et al., 1990]. The constructed anti-preS2 chimeric antibody H69K (IgG1) was shown to have the same affinity for the preS2 peptide as H8, $2.5 \times 10^8\ M^{-1}$ [Jin et al., 1993]. The construction of anti-S chimeric antibody CS131A is described below. The 1B3, H69K, and CS131A were included in in vitro neutralization assays.

To construct the anti-S chimeric antibody CS131A (IgG1), the heavy and light chain variable regions of H67 were fused to the constant regions of human γ1 and κ chains, respectively, and the resulting chimeric heavy and kappa chain cDNAs were inserted between the strong HCMV promoter and polyadenylation/termination signals in pRc/CMV and pCMV-dhfr to yield the heavy and light chain expression plasmids, pRc/CMV-HC-S and pKC-dhfr-S, respectively (Fig. 1). The heavy chain plasmid contains the neomycin-resistant gene, while the light chain plasmid additionally contains the crippled selectable/amplifiable dhfr gene. After the heavy and light chain constructs were cotransfected into dhfr-deficient CHO cells, several resistant cell clones producing the antibody were selected, subjected to successive rounds of MTX selection and screened for the antibody production. Finally, a cell clone (CS131A) with the productivity of 19 μg/10^6 cells/day was selected.

The CS131A cells were grown in serum-free medium and the chimeric antibody was purified from the culture supernatant by an affinity chromatography on Protein G-Sepharose. The size and purity of the purified antibody was confirmed by 10% SDS-PAGE (data not shown). The antigen-binding affinity of the chimeric antibody was determined and compared with that of the parental murine mAb H67. The result showed that they have the same affinity to the HBsAg with approximately $8 \times 10^8\ M^{-1}$ (Fig. 2).

Production and Immunoprecipitation of HBV Particles by Antibodies

To produce infectious HBV particles (*adr* subtype), a replication competent and terminally redundant HBV-plasmid pHBV5.2 was constructed (Fig. 3). The plasmid contains 4 major HBV open reading frames (C, S, P, and X) and another open reading frame C under the control of their autologous regulatory elements. The plasmid DNA was introduced into HepG2 cells and the produced virus particles were isolated from the culture supernatant as described in Materials and Methods. In the same way, the *ayw* subtype of virus was also prepared from the culture supernatant of 2.2.15 cells.

To confirm that the anti-preS1, S2, or S antibody really binds to the HBV particles, immunoprecipitation was undertaken using the *adr* or *ayw* subtype of the virus and each of the antibodies, then the viral DNA was extracted from the immunoprecipitates and analyzed by Southern blot hybridization. The result showed that the relaxed circular (RC) form of HBV DNA was detected in all the precipitates, indicating that all these antibodies bound to both subtypes of HBV particles (Fig. 4). The anti-preS1 antibody (1B3) immunoprecipitated the virus particles less efficiently compared to the anti-preS2 or anti-S antibody. This would be partly due to the fact that the affinity of Protein A to mouse γ1 of 1B3 is lower than to human γ1 of the anti-preS2 or S chimeric antibody [Harlow and Lane, 1988]. Also, the epitope of 1B3 may be less accessible to the surface of the virion, or the affinity of 1B3 may be lower than that of anti-preS2 or S antibody.

In Vitro Infection and Neutralization Assays

Adult human hepatocyte primary culture was infected with the *adr* or *ayw* subtype of HBV. Viral infection and subsequent replication in the human hepatocytes were confirmed by measuring the kinetics of HBsAg secretin by the infected cells during 15 days postinfection, with the medium being changed every day. As shown in Fig. 5A, the secretion rate of the HBsAg increased with infection time and thus the HBsAg concentration on day 15 reached about 28 P/N ratio, indicating that HBV indeed infected the cultured human hepatocytes and replicated in the cells. The viral replication was also evidenced by the presence of the intermediate replicative forms of HBV DNA including covalently closed circular (CCC) form in the infected cells, as shown in the Southern blot analysis (Fig. 5B).

To examine whether the anti-preS1, pre-S2, or S

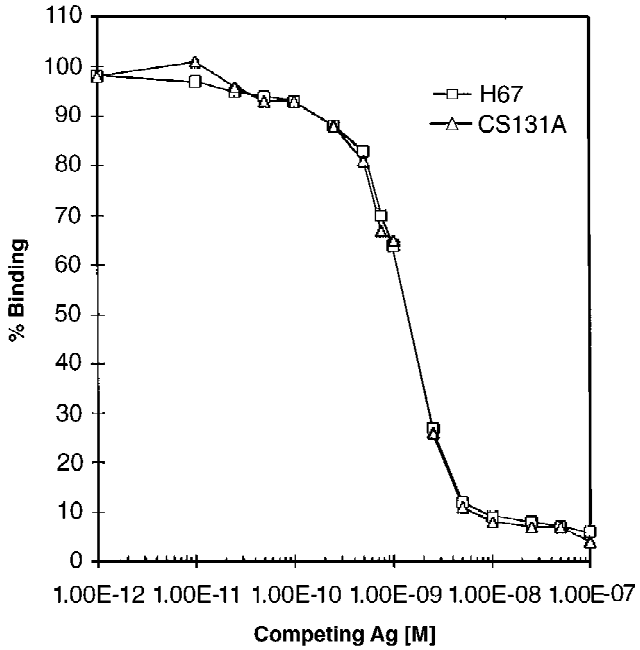

Fig. 2. Affinity determination of H67 and CS131A. Various concentrations of HBsAg were previously incubated with H67 (□) and CS131A (△) in solution first and used as competitors for the bound HBsAg.

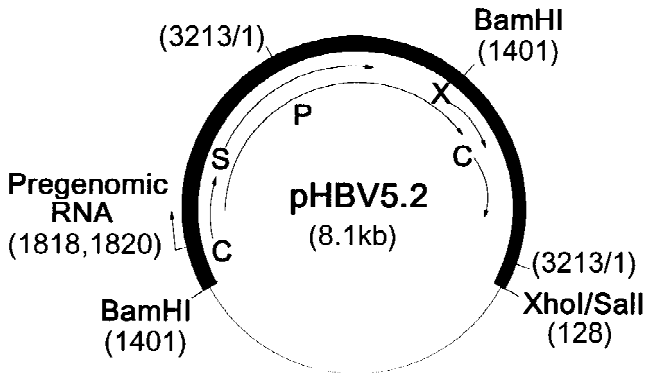

Fig. 3. The map of plasmid pHBV5.2 C, S, P and X represent the four major genes of HBV. The nucleotide positions of HBV genome are shown in parentheses. The arrows indicate the orientation of the transcription.

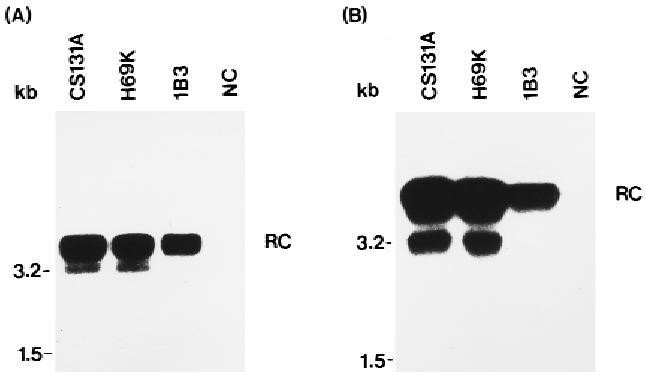

Fig. 4. Immunoprecipitation of HBV particles by the chimeric antibodies H69K, CS131A and murine mAb 1B3. Both *adr* (A) or *ayw* (B) subtype of HBV particles was immunoprecipitated with each antibody bound to Protein A-Sepharose. HBV genome was extracted from the immunoprecipitate and analyzed by Southern blot hybridization using an 1.5% agarose gel. Molecular weight markers are indicated in kilobases on the left. RC represents relaxed circular form of HBV genome. NC means a negative control without using any antibody.

TABLE I. Radioimmunoassay for HBsAg Secretion by the Human Hepatocytes Infected With Antibody-Treated HBV

A. *adr* subtype[a]

Abs μg/ml	HBIG	1B3	H69K	CS131A	Human IgG
5000	0.9	—	—	—	—
1000	1.2	—	—	—	—
100	4.4	1.2	—	—	20
35	—[b]	—	1.2	—	—
10	—	7.4	1.1	1.0	23
1	—	22.4	1.9	1.0	—
0.5	—	—	—	1.2	—
0.1	—	16.7	15.2	3.1	—
0.01	—	21.6	19.8	17.0	—

B. *ayw* subtype[c]

Abs μg/ml	1B3	H69K	CS131A
10	46.8	1.6	1.5
1	60.1	2.0	1.5
0.1	73.1	41.7	4.3
0.01	66.2	60.5	44.0

[a]Control P/N ratio without adding antibody was 27.6. P/N ratio of >2.1 was considered positive.
[b]Not determined.
[c]Control P/N ratio without adding antibody was 53.7. P/N ratio of >2.1 was considered positive.

mAb neutralizes the HBV infection, the virus particles (*adr*) were preincubated with different concentrations of each antibody and the mixture was inoculated onto the hepatocytes. Then, after 15 days, the amount of the secreted HBsAg was assayed. Human IgG and HBIG were used as negative and positive controls, respectively, in this assay. The result was, as summarized in Table IA, that H69K and CS131A completely neutralized the HBV infection at 1 μg/ml and 0.5 μg/ml, respectively, while 1B3 neutralized at 100 μg/ml. HBIG completely neutralized the infection at 1,000 μg/ml and less effectively at 100 μg/ml, while human IgG as a negative control did not show any activity at 100 μg/ml. To verify further the neutralization assay, HBV DNA was isolated from the infected hepatocytes and analyzed by Southern blot hybridization. As shown in Fig. 5B, HBV DNA was not detected from the cells infected with mixture of the virus and CS131A (10, 1, or 0.1 μg/ml), H69K (10 or 1 μg/ml), or HBIG (1,000 μg/ml), while the DNA was detected from the cells infected with the virus and 0.01 μg/ml of CS131A, 0.1 or 0.01 μg/ml of H69K, or 10–0.01 μg/ml of 1B3. Neutralization of the *ayw* subtype of virus by these antibodies was also tested using 2 times higher concentration of the infected hepatocytes compared to that infected with the *adr* subtype of virus and almost the same result was observed, as shown in Table IB. Taken together, the

anti-preS2 and S antibodies neutralized effectively HBV infection in a dose-dependent manner, but the anti-preS1 antibody scarcely exhibited the activity. Comparison of the minimum concentration to exhibit the neutralizing activity between H69K or CS131A and HBIG showed that H69K or CS131A had approximately 1000- or 2000-fold higher specific activity than HBIG.

DISCUSSION

The in vitro neutralization assays showed that the anti-S chimeric antibody CS131A and the anti-preS2 chimeric antibody H69K neutralized effectively the HBV infection, while the anti-preS1 antibody 1B3 scarcely neutralized. These results are consistent with our prediction of their neutralizing activity from the epitope analysis of the antibodies. HBsAg has a number of antigenic specificities but bears the common α antigenic determinant that is present on all subtypes of HBsAg. The common *a* determinant induces virus-neutralizing antibodies in humans [Bhatnager et al., 1982] and as many as 15 epitopes can be distinguished [Wands et al., 1984]. Our anti-S murine antibody H67 is considered to recognize the common *a* determinant because it binds to a disulfide-bond-dependent conformational epitope on the both *ad* and *ay* subtypes of S antigen [Ryu et al., 1994]. In addition, further study showed that H67 partially competes with F5.28.6, which is considered a virus-neutralizing antibody [Petit et al., 1991], in a competition binding assay [Ryu et al., 1996], suggesting that H67 and F5.28.6 may recognize overlapped fine epitopes on the same epitope cluster. From the analyses, we concluded that H67 could be another neutralizing antibody. In case of the anti-preS2 antibody, murine antibody H8 was shown to recognize amino acids 13–24 on the preS2 antigen [Hong et al., 1992] and expected to exhibit the neutralizing activity, since this epitope is known as a virus-neutralizing epitope [Neurath et al., 1986; Itoh, et al., 1986]. On the other hand, 1B3 was shown to recognize the N-terminal 20 amino acids of longer version of HBV preS1 derived from *adr* subtype [Kim et al., 1996] and its neutralizing activity had not been confirmed, while the amino acids 21–47 of preS1 region was known to be responsible for inducing the neutralizing antibody [Neurath et al., 1989]. The present result showed that 1B3 bound to the virus less efficiently and exhibited the lowest neutralizing activity. Taken together, this neutralization assay is very specific and thus useful for testing the neutralizing activity of anti-HBV antibodies.

The neutralization assay showed that the minimum concentration of CS131A or H69K for the neutralizing activity was 0.5 or 1 μg/ml, respectively, as shown in Table IA, suggesting that the neutralizing activity of CS131A may be more potent than that of H69K. Little is known about the mechanism of HBV neutralization, but theoretically the antibody with higher affinity could have more potent neutralizing activity than that with lower affinity, provided that the target antigens are present in the same ratio and recognized with equal accessibility on the surface of virus. The HBV envelope consists of large (containing preS1 + preS2 + S antigens), middle (containing preS2 + S antigens), and major (containing S antigen) proteins, and thus the S antigen is much more present on the virus than preS2 antigen. Also, the affinity (8×10^8 M^{-1}) of CS131A for the S antigen was shown to be higher than that (2.5×10^8 M^{-1}) of H69K for preS2 antigen. Therefore, it seems that more potent neutralizing activity of CS131A is due to the larger number of target antigen and the higher antigen-binding affinity of CS131A compared to H69K.

The 3 surface antigens of HBV were shown to elicit virus-neutralizing and protective antibodies. So far, human mAbs against the S antigen were tested for the protective and neutralizing efficacies [Harada et al., 1989; Ogata et al., 1993]. However, it was reported that antibodies against the preS region play an important role in the clearance and recovery from acute viral infection and can overcome nonresponsiveness to S antigen [Milich et al., 1985; Neurath et al., 1985; Budkowska et al., 1986; Milich et al., 1986; Neurath et al.,

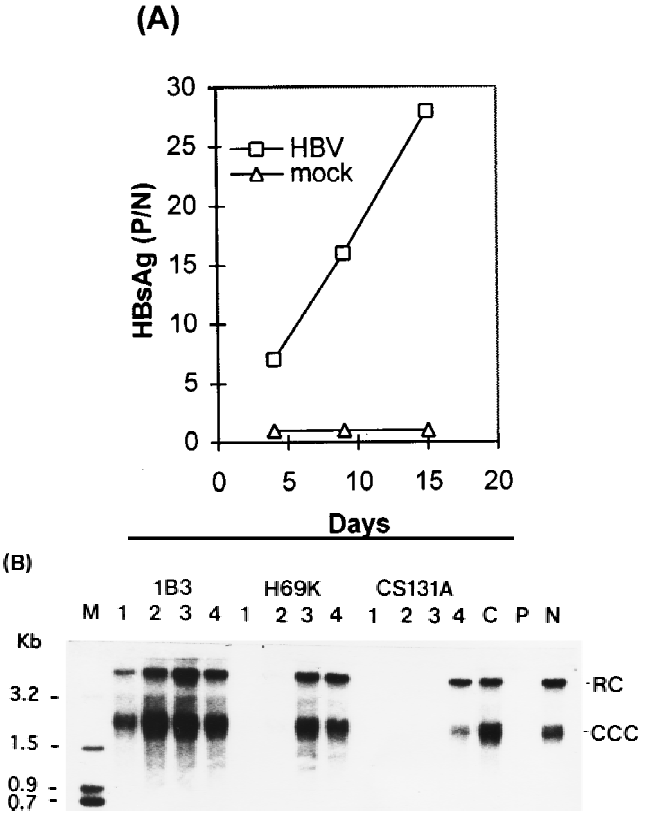

Fig. 5. **A:** Kinetics of HBsAg secretion by mock (△) or HBV (□)-infected human hepatocyte primary cultures. **B:** Southern analysis of the intracellular HBV DNA from the cells infected with the antibody treated HBV. The 3 used antibodies 1B3, H69K, and CS131A are indicated. Lane 1, 10 μg/ml; lane 2, 1 μg/ml; lane 3, 0.1 μg/ml; lane 4, 0.01 μg/ml; lane C, mock; lane P, 1,000 μg/ml HBIG; lane N, 100 μg/ml human IgG. Molecular weight markers are indicated in kilobases on the left. RC and CCC represent relaxed circular and covalently closed circular form of HBV genome, respectively.

1989]. In addition, the antibody to the preS may block efficiently the binding of virus to hepatocyte cell receptor [Machida et al., 1984; Neurath et al., 1986; Pontisso et al., 1989]. Also, a small fraction of virus often escapes neutralization even when a large excess of neutralizing antibody is used [Mandel, 1985]. Therefore, administration of cocktails of the anti-preS1, preS2, and S antibodies to prevent HBV infection will be beneficial. Synergistic neutralization of HBV infection by these antibodies, which has been observed from studies with other virus, for example, human immunodeficiency virus [Potts et al., 1993; Laal et al., 1994; Sujata et al., 1996], may be expected.

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